Evaluation of the Susceptibility of Pathogenic *Candida* Species to Fluconazole

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A fluconazole 25 µg disk diffusion test was used to test 2230 consecutively isolated *Candida* strains from 42 different hospital laboratories in 23 countries. Ninety seven percent of 1634 *Candida albicans* isolates and 83.4% of 596 non-*Candida albicans* isolates were susceptible to fluconazole, applying the proposed breakpoints (≥26 mm for susceptible strains and 18–25 mm for dose-dependent susceptible strains). This is the first hospital laboratory study to evaluate a large number and wide range of sequential *Candida* isolates from patients with all types of hospital infections. The fluconazole disk diffusion test appears to be a low-cost, reproducible, and accurate means of assessing the in vitro susceptibility of *Candida* isolates.

The lack of a reliable standardised antifungal susceptibility test method has complicated the interpretation of many study reports until recently. However, significant progress has been made in the past ten years in developing more practical methods for the susceptibility testing of yeasts. Efforts of the National Committee for Clinical Laboratory Standards (NCCLS) have focused on developing a reference macrobroth minimum inhibitory concentration (MIC) method for testing yeast species (1). Work is still ongoing to improve that assay, which is relatively difficult to perform and read, particularly when large numbers of isolates are tested. However, it does provide a reference for comparison with other methods. Microdilution plate methods have been developed that produce results comparable to those of the NCCLS method and are easier to perform and read (2–5). However, the reading of endpoints still poses problems in both the macrobroth and microbroth assays.

Agar gradient methods have also been developed that appear to provide reproducible, accurate, and more easily read results. These include the E test (6) and disk diffusion test (7, 8). These assays are more practical for use in clinical laboratories. In both the E test and the disk diffusion test, results are reproducible, the tests are easy to set up, they have adequate controls, and the results are read on a precise quantitative scale. The disk diffusion test has the advantages of lower material costs, and relatively easy to read results. Growth inhibition zone diameters in the disk diffusion test also correlate well with the NCCLS reference method MICs, and with the MICs determined in the E test assay (8, 9).

The purpose of this study was to evaluate the susceptibility to fluconazole of a large population of pathogenic *Candida* isolates from a wide range of hospitals and countries using the only standardised disk diffusion test method available at the time the study was started (Unipath, Pfizer, UK).

Materials and Methods. Forty-two hospital laboratories used the agar disk diffusion test (Unipath) to test approximately 50 consecutively isolated yeast strains each, all strains being considered pathogens in the patients from whom they were obtained. A total of 2230 isolates from 23 countries were tested. Each yeast isolate was from a different patient; isolates from all types of infections and body sites were included in the study. Data on the mycological and clinical response of fluconazole-treated patients were collected when possible. Information on the duration of therapy, dose of fluconazole, concomitant medication, primary diagnosis, underlying diseases, and other data usually collected in clinical trials was not considered relevant in this laboratory study. Three quality control strains of *Candida albicans* (Y01–09, Y01–108, Y01–19) were included with each batch of yeast isolates tested. The agar diffusion test was performed exactly as described by the original manufacturer (data on site, Pfizer, USA) using 25 µg fluconazole disks on 4 mm deep High Resolution (HR) agar medium in 90 mm petri plates. The yeast inoculum was prepared by adjusting the density of yeast cells in a normal saline suspension, using a microscope and haemocytometer, to approximately 20 cells/mm^2 in the grid. The final cell density was adjusted to 4 x 10^7 cells/ml. After

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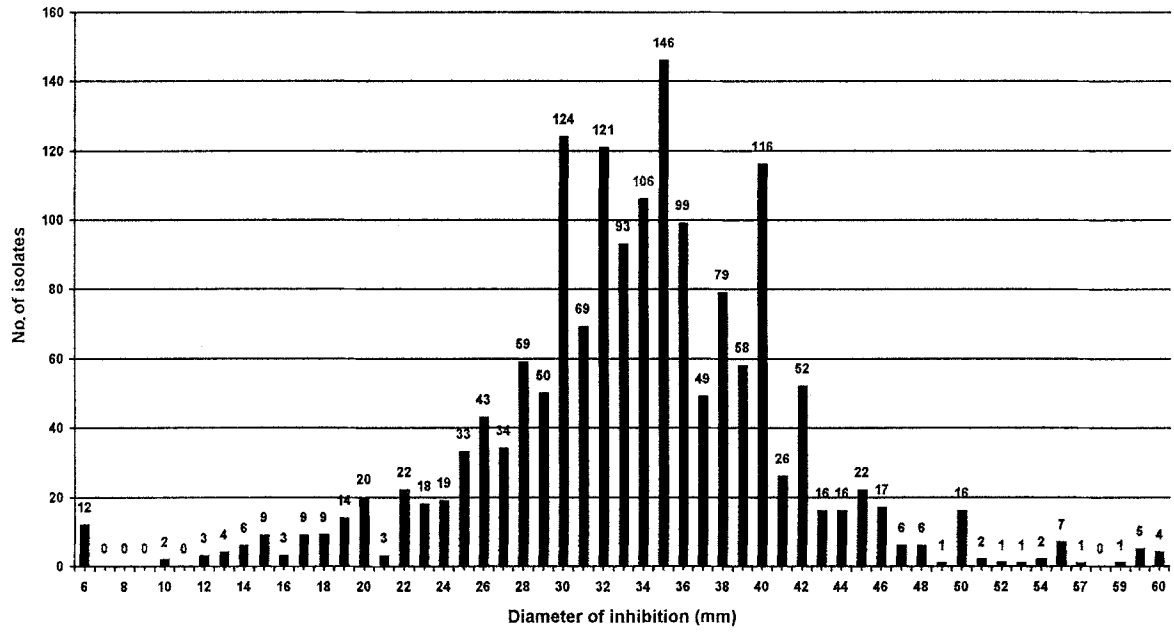

Figure 1: Susceptibility to fluconazole of 1634 isolates of *Candida albicans* as determined by the disk diffusion test on HR medium incubated at 28°C. The proposed breakpoints are: resistant < 18 mm; dose-dependent susceptible 18–25 mm; susceptible ≥ 26 mm.

incubation for 24 h at 28°C, the diameter of the zone of inhibition of yeast growth around the fluconazole disks was measured and recorded. Quality control strains were tested each day the clinical isolates were tested and results recorded; the diameter of their inhibition zones had to fall within the ranges specified.

Because the MICs were not systematically determined for these isolates, MIC correlates cannot be derived from these data. The proposed breakpoints for the disk diffusion test are deducted from statistical analysis of the inhibition zone diameters measured.

Results and Discussion. The results for 1634 *Candida albicans* isolates are shown in Figure 1. They indicate that 97.1% of the *Candida albicans* isolates were susceptible, or dose-dependent susceptible, to fluconazole using a cut-off value of ≥ 18 mm. The isolates displayed a normal bell-shaped distribution with a normality value of 0.96, mean of 33.5 mm, mode of 35 mm, and standard deviation (SD) from the mean of 7.6 mm. One SD from the mean included zone diameters from 26 to 41 mm, and two SDs included zone diameters from 18 to 49 mm; 95% of test results were between two SDs. These SD values provided a statistical basis for proposing ≥ 26 mm and < 18 mm as the susceptibility and resistance breakpoints, respectively. The preliminary resistance breakpoint of < 18 mm, as defined in the protocol, thus remained unchanged. However, the preliminary susceptibility breakpoint of ≥ 30 mm, as defined in the protocol, divided the normal susceptible population near the mean of the zone diameter measurements. The proposed breakpoints separated the susceptible population from the more resistant population, i. e. those falling outside of two SD and representing 2.9% of 1634 isolates. Thus, 97.1% of isolates were determined to be susceptible, or dose-dependent susceptible, applying interpretive criteria based on the proposed NCCLS breakpoints. About 8.4% of the *Candida albicans* isolates was categorised as being dose-dependent susceptible, i.e. the isolates were susceptible to doses greater than 100 mg fluconazole daily.

The susceptibility to fluconazole of 596 isolates of other *Candida* species is shown in Figure 2. As most of these isolates showed growth rates comparable to those of *Candida albicans* on HR medium, these tests were read after 24 hours of incubation at 28°C. When the criteria developed for *Candida albicans* were applied to these isolates, 83.6% were determined to be susceptible or dose-dependent susceptible. Only 16.9% were categorised as dose-dependent susceptible with inhibition zones between 18 and 25 mm; these isolates were susceptible to doses greater than 100 mg fluconazole daily, except for *Candida*

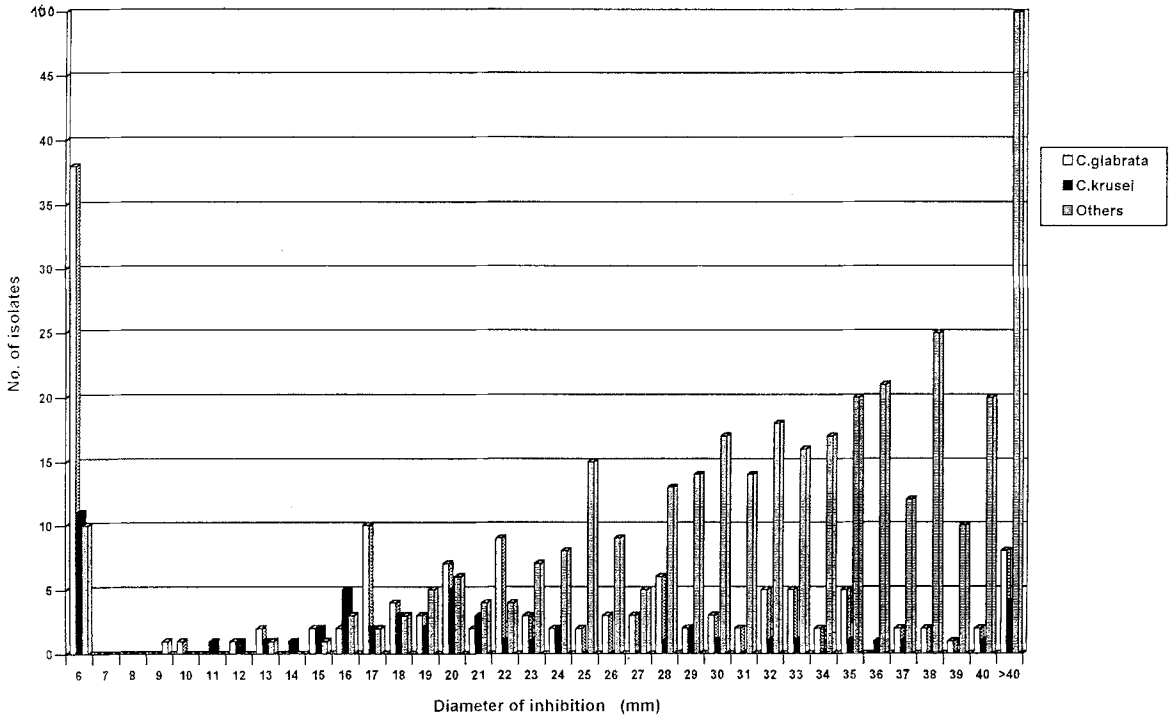

Figure 2: Susceptibility to fluconazole of 596 *Candida* species other than *Candida albicans* including 139 isolates of *Candida glabrata,* 55 isolates of *Candida krusei,* and 402 isolates of other *Candida* species as follows: *C. tropicalis* (197; 6 resistant), *C. parapsilosis* (123; 2 resistant), *C. guillermondii* (22; 1 resistant), *C. inconspicua* (13; 1 resistant), *C. famata* (7; none resistant), *C. humicola* (6; none resistant), *C. lusitaniae* (6; 2 resistant), *C. parakrusei* (6; none resistant), others (22; 4 resistant). For method and breakpoints see legend to Figure 1.

krusei isolates which were considered resistant to fluconazole regardless of the in vitro test result.

Of the non-*Candida albicans* isolates, 32.6% (194/596) were *Candida krusei* or *Candida glabrata.* Although only 16% (98/596) of all non-*Candida albicans* isolates were categorised as being resistant to fluconazole, 44% (24/55) of the *Candida krusei* isolates and 40% (56/139) of the *Candida glabrata* isolates were resistant (Figure 2). Thus, these two species represented 82% (80/98) of all fluconazole-resistant non-*Candida albicans* isolates. Of 101 non-*Candida albicans* isolates categorised as dose-dependent susceptible, 17% were *Candida krusei* and 32% *Candida glabrata.* Of the remaining 397 non-*Candida albicans* isolates categorised as susceptible, only 3.5% were *Candida krusei* and 13% *Candida glabrata.* Thus the disk diffusion test breakpoints proposed for *Candida albicans* also appear to apply to these non-*albicans* species of *Candida,* although clinical studies and clinical experience with these isolates have been much less extensive than with *Candida albicans* (10). *Candida* species that do not produce readily visible growth within the 24 hour incubation period should, for the meantime, be tested by a different method.

Of the three *Candida albicans* quality control strains, *Candida albicans* Y01–19 (NCPF 3726) demonstrated the best reproducibility, 94% (163/174) of the inhibition zone diameter measurements falling between 15 and 21 mm. The two other quality control strains, Y01–108 and Y01–09, yielded a wider range of inhibition zone diameter measurements, with a reproducibility of 75% and 54%, respectively.

Clinical relevance is an important aspect of all drug susceptibility test results. Limited information has been published for any method on the correlation between clinical or mycological responses and test results (7, 9, 11). Rex et al. (9, 11, 12) reported that a strong correlation existed between clinical outcome and fluconazole MIC using the NCCLS M27-T broth method to test *Candida* isolates, and that higher doses could be used against isolates with higher MICs. Isolates with MICs < 64 µg/ml tended to respond well to fluconazole therapy, whereas those with MICs ≥ 64 µg/ml (and smaller zone diameters) tended to respond

poorly. Troillet et al. (7) also showed good correlation between zone diameter results in the fluconazole disk diffusion test and the clinical and mycological outcome in AIDS patients.

The interpretive guidelines defined in this study protocol were preliminary and based on the limited data available from human and animal efficacy studies of fluconazole at the time the protocol was written. Since then, test results for isolates in rigorously controlled clinical protocols with strict patient entry and exclusion criteria have provided data consistent with the results of this study (9, 12). However, clinical efficacy protocols, by design, exclude many patients and pathogens. Consequently, they may provide a different pattern of results than the non-selected sequential pathogens tested in this hospital laboratory study which is the first to evaluate a large number and wide range of sequential *Candida* isolates from patients with all types of hospital infections.

The fluconazole disk diffusion method used in this study proved to be easy to set up and read, and provided test results that were reasonably reproducible in a diverse group of hospital laboratories, as determined by isolate and quality control strain performance. In particular, *Candida albicans* control strain Y01–19 (ATCC 76615/NCPF 3726) yielded an acceptable performance when testing for small zone diameters. The disk diffusion test appears to be a reproducible and accurate means of assessing the susceptibility of a wide range of yeasts in hospital laboratories (10). Materials are readily available at low cost, it is easy to establish controls, and the method is similar to methods commonly used to test bacterial isolates. Technical difficulties were experienced in conjunction with the 28°C incubation temperature, which is unusual for hospital incubators, the haemocytometer inoculum adjustment, and making HR medium. Subsequent to this study, improvements in the disk diffusion test that addressed these technical difficulties were evaluated (8); 250 *Candida* species isolates were thereby tested in parallel by the standard macrobroth dilution assay, fluconazole E test, and 25 μg fluconazole disk diffusion test (using RPMI medium with 2% glucose, swab inoculation of an inoculum of 0.5–2.5 x 10^3 cfu/ml, incubation at 35°C for 24 and 48 h). All three methods were in excellent agreement.

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References

1. National Committee for Clinical Laboratory Standards: Reference method for broth dilution antifungal susceptibility testing of yeast. Tentative standard M27-T. NCCLS, Villanova, PA, 1995.
2. Polanco AM, Rodriguez-Tudela JL, Baquero F, Sanchez-Sousa A, Martinez-Suarez JV: Improved method of determining the susceptibility of *Candida albicans* to fluconazole. Journal of Antimicrobial Chemotherapy 1995, 35: 155–159.
3. Fournier C, Gaspar A, Boillot F, Villard J: Evaluation of a broth microdilution antifungal susceptibility test with a pH indicator: comparison with the broth macrodilution procedures. Journal of Antimicrobial Chemotherapy 1995, 35: 373–380.
4. Rodriguez-Tudela JL, Martinez-Suarez JV: Defining conditions for microbroth antifungal susceptibility tests: influence of RPMI-2% glucose on the selection of endpoint criteria. Journal of Antimicrobial Chemotherapy 1995, 35: 739–749.
5. Espinel-Ingroff A, Rodriguez-Tudela JL, Martinez-Suarez JV: Comparison of two microdilution procedures with the proposed NCCLS reference macro-dilution method M27-P for in vitro testing of fluconazole resistant and susceptible isolates of *Candida albicans*. Journal of Clinical Microbiology 1995, 33: 3154–3158.
6. Colombo J, Barchiesi F, McGough DA, Fotherfill AW, Rinaldi MG: Evaluation of the E-test system versus a microtitre broth method for antifungal susceptibility testing of yeast against fluconazole and itraconazole. Antimicrobial Agents and Chemotherapy 1995, 36: 93–100.
7. Troillet N, Durussel C, Bille J, Glauser MP, Chave JP: Correlation between in vitro susceptibility of *Candida albicans* and fluconazole resistant oropharyngeal candidiasis in HIV-infected patients. European Journal of Clinical Microbiology & Infectious Diseases 1993, 12: 911–915.

8. Barry A, Brown S: Fluconazole disk diffusion procedure for determining susceptibility of *Candida* species. Journal of Clinical Microbiology 1996, 34: 2154–2157.
9. Rex J, Pfaller MA, Barry AL, Nelson PW, Webb CD: Antifungal susceptibility testing of isolates from a randomized multicenter trial of fluconazole versus amphotericin B as treatment of non-neutropenic patients with candidemia. Antimicrobial Agents and Chemotherapy 1995, 39: 40–44.
10. van't Wout JW: Fluconazole treatment of candidal infections caused by non-*albicans Candida* species. European Journal of Clinical Microbiology & Infectious Diseases 1996, 15: 238–242.
11. Ghannoum M, Rex JH, Galgiani J: Susceptibility testing of fungi: current status of correlation of in vitro data with clinical outcome. Journal of Clinical Microbiology 1996, 34: 489–495.
12. Rex J, Pfaller MA, Galgiani JN, Bartlett MS, Espinel-Ingroff A, Ghannoum MA, Lancaster M, Odds FC, Rinaldi MG, Walsh TJ, Barry A: Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of in vitro – in vivo correlation data for fluconazole, itraconazole, and *Candida* infections. Clinical Infectious Diseases 1997, 24: 235–247.

Nosocomial Outbreak of *Clostridium difficile* Diarrhea in a Pediatric Service

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An outbreak of nosocomial diarrhea that occurred in a pediatric orthopedic service between 1 December 1993 and 15 April 1994 is reported. A total of 37 patients (mean age, 9.6 years; range, 2 months–19.3 years) were involved in the outbreak, including six patients with bacteriologically documented *Clostridium difficile* infection. A multivariate analysis identified lincomycin treatment for at least three days as the only significant risk factor. Stool samples from four asymptomatic patients were also positive for *Clostridium difficile* and its cytotoxins. Isolates from all patients belonged to serogroup C, were highly resistant to lincomycin, and exhibited the same restriction pattern by pulsed-field gel electrophoresis. The outbreak ended after treatment with lincomycin was discontinued and hygiene control measures were implemented.

Clostridium difficile is the most common agent of pseudomembranous colitis and antibiotic-associated diarrhea (1). It has been implicated in many outbreaks of diarrhea affecting hospitalized adult patients (2, 3). Hospital acquisition of *Clostridium difficile* has also been demonstrated in a pediatric population (4, 5). However, there have been few reports and no epidemiological investigations of nosocomial outbreaks of *Clostridium difficile*-associated diarrhea in pediatric wards (6). We describe an outbreak of diarrhea in a pe-

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